

Campylobacter species occurrence within internal organs and tissues of commercial caged Leghorn laying hens

N. A. Cox,*¹ L. J. Richardson,* R. J. Buhr,* and P. J. Fedorka-Cray†

*USDA, Agricultural Research Service, Poultry Microbiological Safety Research Unit,
and †USDA, Agricultural Research Service, Bacteriological Epidemiology and Antimicrobial
Resistance Research Unit, Russell Research Center, Athens, GA 30605

ABSTRACT *Campylobacter* spp. are frequently present in the intestinal tract and internal tissues of broiler breeder and broiler chickens. *Campylobacter* spp. ecology in commercial Leghorn laying hens has not been extensively studied. The objectives of the current study were to determine 1) *Campylobacter* spp. presence in the reproductive tract, lymphoid organs, liver-gallbladder, and ceca of commercial Leghorn laying hens; 2) species of *Campylobacter* present; and 3) antimicrobial resistance pattern of *Campylobacter* isolates. In study 1, three flocks ranging from 94 to 105 wk of age were sampled from a commercial laying complex. In study 2, two flocks, 82 and 84 wk of age, were sampled from a separate complex. Hens were killed, defeathered, aseptically necropsied, and the spleen, liver-gallbladder, ovarian follicles, and upper (infundibulum, magnum, and isthmus) and lower (shell gland and vagina) reproductive tracts were aseptically removed before the ceca. Samples were packed on ice and transported to the laboratory for evaluation. For speciation, a standard BAX real-time PCR method was used while susceptibility testing was performed using US National An-

timicrobial Resistance Monitoring System (NARMS) standards and recommended quality control organisms. Isolates were examined for susceptibility using a semi-automated testing system (Sensititer) to the following 9 antimicrobials: azithromycin, clindamycin, ciprofloxacin, erythromycin, florfenicol, gentamicin, nalidixic acid, telithromycin, and tetracycline. In study 1, the isolation rate was 13, 67, 53, 3, 13, and 57% from the ovarian follicles, lower reproductive tract, upper reproductive tract, spleen, liver-gallbladder, and ceca, respectively. In study 2, the isolation rate was 17, 43, 33, 20, 17, and 73% from the ovarian follicles, lower reproductive tract, upper reproductive tract, spleen, liver-gallbladder, and ceca, respectively. Overall, 50% of isolates were *Campylobacter jejuni*, 49% *Campylobacter coli*, and 1% *Campylobacter lari*. In study 1, all of the isolates were pan-susceptible. In study 2, thirty-seven percent of the isolates were resistant to tetracycline. Commercial table egg laying hens housed in colony cages on wire floors had diverse *Campylobacter* spp. recovered from different tissues and these isolates were not resistant to a broad range of antimicrobials.

Key words: *Campylobacter*, antimicrobial resistance, caged laying hen, internalization, ceca

2009 Poultry Science 88:2449–2456
doi:10.3382/ps.2009-00195

INTRODUCTION

For approximately 4 decades, the genus *Campylobacter* has had increased focus as a threat to food safety because of the rise in gastroenteritis in humans caused by consumption or handling of foods contaminated with the organism. Approximately 70% of human illnesses due to *Campylobacter* spp. are caused by the consumption or handling of raw or undercooked poultry or poultry products (Mead et al., 1999; Friedman et al., 2000). In an analysis of foodborne outbreak data from 1988 to 2007 in which *Campylobacter* spp. were the eti-

ological agent, 36% of outbreaks were linked to poultry products (Greig and Ravel, 2009). The infectious dose for campylobacters can be a few hundred cells (Black et al., 1988). Even though *Campylobacter* spp. are sensitive to drying, high oxygen concentration, and low pH (≤ 4.7), they are still a major cause of human bacterial gastroenteritis. In the United States, approximately 2.5 million people are infected each year due to consumption of foods containing campylobacters (Mead et al., 1999). In 2008, the number of human infections with *Campylobacter* spp. was estimated at 12.68 per 100,000 people in the United States (Anonymous, 2009). Four species (*Campylobacter jejuni*, *Campylobacter coli*, *Campylobacter lari*, and *Campylobacter upsaliensis*) are known as thermotolerant campylobacters and are clinically significant due to their association as dominant

©2009 Poultry Science Association Inc.

Received April 17, 2009.

Accepted July 11, 2009.

¹Corresponding author: nelson.cox@ars.usda.gov

causative agents of human campylobacteriosis (Blaser et al., 1982; Jacobs-Reitsma, 2000; Keener et al., 2004). *Campylobacter jejuni* is the predominant species that causes bacterial gastroenteritis in the United States and in many other developed countries, with *C. coli* being second (Lastovica, 2006).

The number of contaminated broilers accounts for the high *Campylobacter* spp. prevalence in poultry processing plants and on processed carcasses (Jacobs-Reitsma, 2000; Allen et al., 2007). In 2002, 35 US broiler flocks were evaluated and *Campylobacter* was recovered from 32 out of 35 of the flocks (Cox et al., 2002a). The average prevalence rate of infected flocks in a separate survey comparing multiple studies was 44 to 59% (Humphrey et al., 2007; Nauta and Havelaar, 2008). In 2007, 8 US poultry processing plants were evaluated and the overall prevalence rate of broiler carcasses contaminated with *Campylobacter* spp. before entering the chiller was 99% and 84% of carcasses were positive postchill (Richardson et al., 2009). The average *Campylobacter* spp. prevalence rate on raw chicken comparing multiple studies was 57% at retail with a range of 23 to 100% (Humphrey et al., 2007). In a separate study, comparing information from 7 different studies and evaluation of 1,167 samples, *Campylobacter* prevalence on retail poultry was determined to be 71.5% in the United States (Suzuki and Yamamoto, 2009). The high colonization of poultry, presence on retail poultry, and the resultant clinical infection in humans have prompted several investigations focused upon identifying and subsequently eliminating sources of *Campylobacter* spp. contamination in poultry flocks (Solomon and Hoover, 1999).

In chickens, the main colonization site of *Campylobacter* spp. is the mucus layer within the lower intestinal tract overlaying the epithelial cells in the ceca and cloacal crypts (Meinersmann et al., 1991). *Campylobacter* spp. once colonized within the intestines can be found in levels up to 10^9 cfu/g of fecal content (Altekruse et al., 1999). Current studies have found that *Campylobacter* spp. are present in various organs and tissues within commercial broilers and broiler breeders. In commercial broiler breeder hens, *C. jejuni* and *C. coli* were isolated from mature and immature ovarian follicles and from all segments of the reproductive tract (Jacobs-Reitsma, 1997; Carmarda et al., 2000; Buhr et al., 2002; Cox et al., 2005). *Campylobacter* spp. have been recovered from primary (thymus) and secondary (spleen) lymphoid organs as well as from the liver-gallbladder of commercial broilers and broiler breeder laying hens (Cox et al., 2006a, 2007).

Antibiotics have been used to treat animals and humans since their discovery. Erythromycin is a drug used to treat humans for gastrointestinal campylobacteriosis but fluoroquinolone and ciprofloxacin are also effective (Blaser, 1995). The increased resistance of this organism to these drugs could become a threat to public health (Randall et al., 2003). Therefore, it is important to evaluate *Campylobacter* spp. in different poultry op-

erations to determine differences in antimicrobial resistance patterns (Ge et al., 2003; Randall et al., 2003; Price et al., 2005). Randall et al. (2003) found that out of 443 poultry isolates, 3.8% of *C. coli* and 3.3% of *C. jejuni* had resistance to 3 or more antibiotics. In a study by Price et al. (2005), they found that 84% of the chicken products tested were *Campylobacter*-positive and fluoroquinolone-resistant strains were detected in 40% of the isolates. In a comparison study, the prevalence of ciprofloxacin-resistant *Campylobacter* was 13% in 1997 and 19% in 2001 and erythromycin-resistant *Campylobacter* was unchanged during that same period at 2% (Gupta et al., 2004).

Based on surveys by public health authorities in industrialized countries and available epidemiological information, table eggs are not regarded as a significant vehicle of foodborne *Campylobacter* infection for consumers (Shane and Stern, 2003). Greig and Ravel (2009) analyzed foodborne outbreak data from 1988 to 2007 reported internationally for source attribution and 1.6% of *Campylobacter* outbreaks originated from eggs. In regard to total egg outbreaks, *Campylobacter* contributed to 0.6% of the 584 outbreaks and 97.4% were attributed to *Salmonella* (Greig and Ravel, 2009). The ecology of *Campylobacter* spp. within commercial caged laying hens has not been completely investigated due to the low rate of *Campylobacter* outbreaks associated with eggs. In addition, the presence of *Campylobacter* spp. within internal organs and tissues has not been investigated. A few studies have evaluated the natural presence of *Campylobacter* spp. in commercial caged laying hens and table eggs (Doyle, 1984; Papadopoulos, 1997; Adesiyun et al., 2005; Jones and Musgrove, 2007). *Campylobacter jejuni* can colonize and persist in the ceca of caged Leghorn hens for at least 42 wk (Doyle, 1984). Surveying pooled commercial egg samples from 23 poultry layer farms located in Trinidad, *Campylobacter* spp. were isolated from 1% of samples (Adesiyun et al., 2005). *Campylobacter* spp. were recovered from 0.5% of commercial table eggs tested in the United States (Jones and Musgrove, 2007).

Campylobacter spp. prevalence within commercial table eggs is low, whereas the prevalence and ecology within commercial laying flocks has not been fully elucidated. Susceptibility of *Campylobacter* isolates originating from commercial laying hen operations to certain antimicrobials is also unknown. The objectives of the study were to determine *Campylobacter* spp. prevalence in reproductive tracts, lymphoid organs, liver-gallbladder and ceca of commercial caged Leghorn laying hens, *Campylobacter* species present, and antimicrobial resistance profile of the isolates.

MATERIALS AND METHODS

Experimental Design

To evaluate the presence of *Campylobacter* spp. in commercial in-line caged laying hens ($n = 60$), 2 studies

were conducted at 2 separate commercial complexes. In study 1, three flocks (10 birds/flock), 98, 102, and 104 wk of age, were sampled from 1 laying complex. Flocks 1, 2, and 3 were sampled in May, June, and July, respectively. In study 2, two flocks (15 birds/flock), 82 and 84 wk of age, were sampled from a separate laying complex. Flock 4 was sampled in September, whereas flock 5 was sampled in October. Hens were killed, defeathered, necropsied, and the spleen, liver-gallbladder, ovarian follicles (mature and immature) and upper (infundibulum, magnum, and isthmus) and lower (shell gland and vagina) reproductive tracts were aseptically removed in sequential order before the ceca using aseptic techniques described previously (Cox et al., 2006a). Samples were packed on ice and transported to the laboratory for evaluation.

Laboratory Procedure

The spleen, live-gallbladder, ovarian follicles, upper and lower reproductive tracts, and ceca were individually weighed. The samples within plastic bags were then macerated with a rubber mallet to ensure that the contents of the samples were exposed. Bolton's enrichment broth (Acumedia Manufacturers Inc., Baltimore, MD, containing 5% lysed horse blood) was added to the sample bags at a ratio of 3 times the weight of the sample and then stomached (Technar Company, Cincinnati, OH) for 1 min. A 0.1-mL solution of each sample was then direct-plated by streaking in duplicate onto campy-cefex agar plates (Acumedia Manufacturers, Inc.) and incubated in a microaerobic atmosphere (5% O₂, 10% CO₂, 85% N; Airgas, Athens, GA) at 42°C for 48 h. The enrichment samples were also incubated in a microaerobic atmosphere at 42°C for 48 h. A 0.1-mL solution from the enrichment broth of samples negative by direct plating was streaked in duplicate onto campy-cefex agar plates (Acumedia Manufacturers Inc.) and incubated microaerobically at 42°C for 48 h. From plates, suspect colonies (1 to 3) were picked and for presumptive identification, microscopic observation of characteristic spiral cells and tumbling motility in wet mount preparations was performed. The colonies were confirmed *Campylobacter* through latex agglutination (Panbio Inc., Columbia, MD). Isolated colony-forming units were then picked and streaked for isolation onto blood agar (Remel, Lenexa, KS) and incubated microaerobically at 42°C for 48 h. Pure cultures were then placed onto bacterial preservers (treated beads in a cryopreservative fluid) and stored in an ultralow freezer at -80°C until identification of species along with antimicrobial-resistant profile could be performed.

Campylobacter Speciation

Isolates were obtained from the -80°C freezer, placed onto campy-cefex agar, and incubated for 48 h at 42°C in a microaerobic condition. The isolates were then

picked and placed onto blood agar plates and incubated at 42°C for 24 h. Template DNA was prepared by picking 3 to 4 colonies from a plate using a sterile disposable plastic loop and suspending these cells in 200 µL of Qualicon lysis buffer in 1.1-mL plastic tubes (Simport BioTubes, Simport Plastics, Beloeil, Quebec, Canada). A proteinase solution (Qualicon) was added (12.5 µL/mL) to the lysis buffer just before use. The cell suspensions were vortexed briefly and then heated for 20 min at 37°C in a digitally controlled heat block (TruTemp DNA Microheating System, Robbins Scientific, Sunnyvale, CA) to lyse the cells. The tubes were then transferred to a 95°C heat block for a 10-min incubation to inactivate the proteinase. After heat treatment, the lysates were cooled in a 4°C block and either used directly in the BAX system (Dupont Qualicon, Wilmington, DE) or stored frozen at -20°C until needed.

For real-time PCR assay, the reactions were assembled by adding 30 µL of the prepared lysate solutions to 200-µL strip tubes (Dupont Qualicon) held in a 4°C cold block. Each tube held a reagent tablet containing *Taq* DNA polymerase, deoxynucleoside triphosphate, and 2 forward and reverse primer sets (proprietary to Dupont Qualicon), one specific for *C. jejuni*, one specific for *C. coli*, and one specific for *C. lari*. The sealed PCR tubes were then placed in the BAX system Q7 instrument for speciation.

Antimicrobial Susceptibility Testing

Susceptibility testing of *Campylobacter* isolates was achieved using the protocol established for the US National Antimicrobial Resistance Monitoring System for Enteric Bacteria. The *Campylobacter* panel, providing serial dilutions of each antimicrobial and a Sensititer semiautomated system (Trek Diagnostic Systems Inc., Cleveland, OH), was used according to directions of the manufacturer. The *Campylobacter* isolates were obtained from -80°C storage and subcultured on blood agar plates (Remel) then incubated microaerobically at 42°C for 48 h. *Campylobacter* colonies were suspended in Mueller-Hinton broth (Trek Diagnostic Systems Inc.) until suspension turbidity was adjusted to match a 0.5 McFarland standard. From a 0.5 McFarland suspension, 100 µL was transferred into 11 mL of Mueller-Hinton broth containing lysed horse blood (Trek Diagnostic Systems Inc.) and used to inoculate the 96-well panel to give a final concentration of 10⁵ cfu/mL. *Campylobacter* panels included a control well with no antimicrobial drug. All panels were incubated in a microaerobic environment using anaerobe jars at 37°C for 48 h. Quality control ATCC strain *C. jejuni* 33560 was tested to confirm susceptibility to all of the antimicrobials at each testing. The minimum inhibitory concentration for each antimicrobial was read as the first panel well in which no growth was visible. The antimicrobials tested and the resistance breakpoints (minimum inhibitory concentrations) are shown in Table 1.

Table 1. Minimum inhibitory concentrations for antimicrobial susceptibility testing of the *Campylobacter* isolates recovered from Leghorn laying hens

Antimicrobial	Resistant breakpoint ¹ (μg/mL)
Azithromycin	≥8
Ciprofloxacin	≥4
Clindamycin	≥8
Erythromycin	≥32
Florfenicol	≥4
Gentamicin	≥8
Nalidixic acid	≥64
Telithromycin	≥16
Tetracycline	≥16

¹Minimum inhibitory concentrations determined via broth microdilution methods according to National Committee on Clinical Laboratory Standards standards or by breakpoint interpretations used in the National Antimicrobial Resistance Monitoring System.

Statistical Analysis

Data are represented as the number of positive samples over the total number of samples taken. The χ^2 test for independence (InStat 3.0, GraphPad Software Inc., San Diego, CA) was used to evaluate differences in *Campylobacter* spp. prevalence by flock, sample site, and complex. Fisher's exact test (InStat 3.0, GraphPad Software Inc.) was used whenever the expected frequencies fell below 5%. Significance of data was set at $P \leq 0.05$.

RESULTS

The isolation rate of *Campylobacter* spp. from the ovarian follicles, lower reproductive tract, upper reproductive tract, spleen, liver-gallbladder, and ceca from laying hens from the 2 commercial laying complexes is shown in Table 2. No significant ($P > 0.05$) difference was observed between the 2 commercial laying operations in the total number of *Campylobacter* isolates re-

covered. From the 30 birds sampled from 1 commercial laying operation, 63 out of 180 samples were positive for *Campylobacter* spp. From the 30 birds from the second operation, 53 out of 180 samples were positive for *Campylobacter* spp. Significantly ($P < 0.05$) more *Campylobacter* isolates were recovered from the upper and lower reproductive tract along with the ceca compared with the spleen, liver-gallbladder, and ovarian follicles. Table 3 shows the proportion of hens that had *Campylobacter* spp. in multiple sample sites. Significant ($P < 0.05$) difference was observed in the number of sample sites within a hen where *Campylobacter* spp. were recovered. In most of the *Campylobacter*-positive hens, *Campylobacter* spp. were present in only 1 to 3 sample sites. In a few birds, *Campylobacter* spp. were present in 4 to all 6 sample sites.

The proportion of *Campylobacter* spp. recovered within the internal tissue and organs of the laying hens is shown in Table 4. All 3 species evaluated using the BAX system (*C. jejuni*, *C. coli*, and *C. lari*) were isolated and no other species were present. Significantly ($P < 0.05$) more *C. jejuni* and *C. coli* were recovered from the sample sites compared with *C. lari*. No significant ($P > 0.05$) difference was observed between *C. jejuni* and *C. coli* isolation. Also, no significant ($P > 0.05$) differences were observed in the type of species recovered between commercial complexes. The most observed *Campylobacter* species was *C. jejuni*, then *C. coli* and only 1 *C. lari* isolate. Sixty-three out of 125 isolates were *C. jejuni* and 61 out of 125 were *C. coli*. Multiple *Campylobacter* species were observed in certain sampling sites. Co-colonization of *C. jejuni* and *C. coli* was observed 5 times in the lower reproductive tract and 2 times in the upper reproductive tract and ceca from a single hen. Thirteen of the hens were colonized with multiple *Campylobacter* species and *C. jejuni* and *C. coli* were isolated from 12 of the hens and 1 hen with *C. jejuni* and *C. lari*.

Table 2. *Campylobacter* spp. prevalence in the internal organs and tissues of Leghorn laying hens from 2 commercial laying operations

			Sample site ⁴					
Flock ¹	Age ²	Month ³	Spleen	L/G ⁵	Follicle ⁶	UR ⁷	LR ⁸	Ceca
1	98	May	0/10	0/10	0/10	5/10	6/10	2/10
2	102	June	0/10	1/10	1/10	5/10	6/10	7/10
3	104	July	2/10	3/10	3/10	7/10	8/10	7/10
4	82	September	1/15	1/15	1/15	4/15	7/15	8/15
5	84	October	3/15	6/15	4/15	7/15	3/15	8/15
Total			6/60 ^b	11/60 ^b	9/60 ^b	28/60 ^a	30/60 ^a	32/60 ^a

^{a,b}Within a row, means with different letters are significantly different ($P < 0.05$, χ^2 test for independence).

¹Flocks 1 to 3 originated from 1 commercial in-line table egg operation and flocks 4 and 5 originated from a separate commercial in-line operation.

²Age represented in weeks of age.

³Month sampled for each flock.

⁴Prevalence represented as the number of *Campylobacter*-positive samples over total number of samples taken.

⁵Liver and gallbladder composite sample.

⁶Immature and mature ovarian follicle composite sample.

⁷Upper reproductive tract consisting of the infundibulum, magnum, and isthmus.

⁸Lower reproductive tract consisting of the shell gland and vagina.

Table 3. Number of birds that had *Campylobacter* spp. in various sample sites

Flock	Number of sample sites ¹						
	Negative	1	2	3	4	5	6
1	0	1	4	3	0	0	2
2	2	2	2	3	0	1	0
3	6	5	4	0	0	0	0
4	1	8	4	2	0	0	0
5	2	5	3	2	2	1	0
Total birds	11/60 ^{ab}	21/60 ^a	17/60 ^{ab}	10/60 ^b	2/60 ^c	2/60 ^c	2/60 ^c

^{a-c}Within a row, means with different letters are significantly different ($P < 0.05$, χ^2 test for independence. Fisher's exact test was used whenever the expected frequencies fell below 5%).

¹Evaluation of the number of birds that had *Campylobacter* spp. present in sites ranging from zero sites to all 6 sites.

The number of *Campylobacter* isolates that exhibited resistance to each of the 9 antimicrobials is shown in Table 5. *Campylobacter* isolates recovered from the 2 commercial laying operations did not have a broad range of resistance to the tested antimicrobials. From flocks 1, 2, and 3 originating from 1 commercial table egg complex, all isolates were pan-susceptible to the 9 tested antimicrobials. In flock 2, a *C. jejuni* isolate had intermediate resistance to tetracycline and from flock 3, a *C. lari* isolate had intermediate resistance to nalidixic acid. Both of these isolates originated from the upper reproductive tract. From flocks 4 and 5 originating from a different commercial table egg complex, most of the isolates were pan-susceptible to all of the antimicrobials tested except for tetracycline. In flock 4, two *C. jejuni* isolates were resistant to tetracycline and one originated from the ceca and the other from the upper reproductive tract of different hens. The majority of resistant isolates to tetracycline was observed in flock 5 with 52% of isolates recovered within birds exhibiting resistance. Fourteen *C. jejuni* isolates were resistant to tetracycline with 1, 2, 2, 2, 3, and 4 originating from the lower reproductive tract, upper reproductive tract, spleen, liver-gallbladder, ovarian follicles, and ceca, respectively. Two *C. coli* exhibited resistance to tetracycline and one was from the upper reproductive tract and the other from the lower reproductive tract within different hens.

DISCUSSION

It is apparent that *Campylobacter* spp. can be found within primary and secondary lymphoid organs along with the reproductive and digestive tract at a high prevalence rate in Leghorn laying hens originating from commercial facilities. The prevalence rate between the 2 commercial egg laying operations was similar along with the types of *Campylobacter* species recovered. Interestingly, even though there are considerable differences in the management and husbandry practices of Leghorn laying hens compared with broilers and broiler breeder hens (i.e., caged vs. litter vs. slat-litter), the isolation rate was similar between these operation types. In the broiler, *C. jejuni* and *C. coli* are naturally recovered from 20% of the spleen, 17% of the liver-gallbladder, and up to 32% of the unabsorbed yolks of commercial broilers at market age (Cox et al., 2006b, 2007). Naturally occurring *Campylobacter* spp. were recovered from 26% of the thymuses, 19% of the spleens, and 9% of the liver-gallbladders in commercial broiler breeder hens (Cox et al., 2006a). Cox et al. (2005) isolated *Campylobacter* spp. naturally from 26% of the mature and 12% of the immature ovarian follicles of commercial broiler breeder hens. Buhr et al. (2002) found that an increased number of positive samples was observed as sampling moved down the different segments of the reproductive tract toward the vagina and concluded that

Table 4. Number of *Campylobacter jejuni*, *Campylobacter coli*, and *Campylobacter lari* isolates recovered by sample site from Leghorn laying hens from 2 commercial laying operations

Species	Sample site ¹					
	Spleen	L/G ²	Follicle ³	UR ⁴	LR ⁵	Ceca
<i>C. jejuni</i>	4	5	5	14	16	19
<i>C. coli</i>	2	6	4	15	19	15
<i>C. lari</i>	0	0	0	1	0	0

¹Prevalence represented as the number of *Campylobacter*-positive samples.

²Liver and gallbladder composite sample.

³Immature and mature ovarian follicle composite sample.

⁴Upper reproductive tract consisting of the infundibulum, magnum, and isthmus.

⁵Lower reproductive tract consisting of the shell gland and vagina.

Table 5. Results from the susceptibility testing of 125 *Campylobacter* isolates recovered from commercial caged laying hens

Antimicrobial	Pattern ¹	Antimicrobial ²								
		AZ	CI	CM	EM	FL	GM	NA	TEL	TC
Azithromycin	S	125	125	125	125	125	125	124	125	106
Ciprofloxacin	I	0	0	0	0	0	0	1	0	1
Clindamycin	R	0	0	0	0	0	0	0	0	18

¹S: susceptible; I: intermittent; R: resistance.

²The antimicrobials tested and the resistance breakpoints (minimum inhibitory concentrations) were as follows: AZ: azithromycin, ≥ 8 g/mL; CI: ciprofloxacin, ≥ 4 g/mL; CM: clindamycin, ≥ 8 g/mL; EM: erythromycin, ≥ 32 g/mL; FL: florfenicol, ≥ 4 g/mL; GM: gentamicin, ≥ 8 g/mL; NA: nalidixic acid, ≥ 64 g/mL; TEL: telithromycin, ≥ 16 g/mL; and TC: tetracycline, ≥ 16 g/mL.

fecal retrograde contamination could be playing an important role in this trend. In a 2002 study, over 9% of semen samples from commercial broiler breeder rosters were found to naturally be contaminated with *Campylobacter* spp. as high as $3 \log_{10}$ cfu/mL (Cox et al., 2002b). These data suggested that rooster semen may serve as a vehicle for transmitting *Campylobacter* spp. to the reproductive tract of the broiler breeder hen. However, in this present study, there was no rooster to hen interactions.

In 1992, approximately 99% of the clinical cases in the United States were found to be *C. jejuni*, whereas *C. coli* comprised the other 1% of clinical cases (Tauxe, 1992). In a more recent study, 95% of human isolates were found to be *C. jejuni*, 4% *C. coli*, and 1% were other *Campylobacter* species (Gupta et al., 2004). From the National Antimicrobial Resistance Monitoring System surveillance data from 1999 to 2004, it was discovered that out of 2,037 isolates obtained from chicken rinse samples, 66% of the isolates were *C. jejuni* and 34% of the isolates were *C. coli*. From broiler breeder hens, 53% of the isolates recovered were *C. jejuni* and the other 47% of isolates were *C. coli* (Cox et al., 2006a). *Campylobacter jejuni*, *C. coli*, and *C. lari* were isolated from the Leghorn laying hens and the isolation rate was similar to that found in the above studies. However, it was not determined in any of the studies if certain laboratory methodology procedures (i.e., gas mixture during incubation) may have preferentially selected for recovery of *C. jejuni* and *C. coli* while excluding the chances of recovering other species (Lastovica, 2006). *Campylobacter jejuni* and *C. coli* were not found exclusively in a Leghorn laying hen sample site and colonization of internal organs does not appear to be species related.

In Leghorn laying hens, a wide range of resistance to antimicrobials was not observed. Antimicrobial-resistant strains were not limited to 1 sampling site and were found throughout different tissues within a bird. Tetracycline-resistant *C. jejuni* and *C. coli* were present in 2 flocks originating from 1 commercial laying complex with the majority of the isolates coming from one of the flocks. An increase in tetracycline resistance in *C. jejuni* and *C. coli* strains has been observed in

recent years (Luangtongkum et al., 2008). Ge et al. (2003) found that out of 378 *Campylobacter* isolates, resistance to tetracycline was most common at 82%, then doxycycline (77%), erythromycin (54%), nalidixic acid (41%), and ciprofloxacin at 35%. Tetracycline is a broad-spectrum antibiotic that is widely used in human and veterinary medicine (Avrain et al., 2004). The main mechanisms of tetracycline resistance in *Campylobacter* species is a ribosomal protection protein Tet(O) (Taylor and Courvalin, 1988). Tetracycline-resistant *Campylobacter* have been recovered from organic and other production systems in which no antibiotics have been used, which indicates that prior or current use may not be a defining attribution to resistance (Piddock et al., 2000; Luangtongkum et al., 2006). Even though broad-range resistance was not observed with Leghorn laying hen isolates, *Campylobacter* resistance to multiple antibiotics has been observed in broiler operations (Fallon et al., 2003; Son et al., 2007; Gyles, 2008). To better elucidate *Campylobacter* antimicrobial resistance in poultry, the majority of information acquired has been from broiler or turkey carcasses. Further evaluation of the antimicrobial resistance patterns of *Campylobacter* isolates from several different types of poultry rearing and processing environments could help better define the epidemiology and ecology of antimicrobial resistance in poultry.

Strain diversity within *Campylobacter* spp. has been well documented (Meinersmann et al., 1997; Rivoal et al., 2005; Hiett et al., 2007). Genetic characterization of the isolates was not performed, but when completed, this may provide useful knowledge on the epidemiology and ecology of these *Campylobacter* isolates. *Campylobacter* spp. can adhere to and invade chicken intestinal epithelial cells (Hanel et al., 2004; Byrne et al., 2007). *Campylobacter* strains that were more invasive were recovered from the liver of the chickens (Byrne et al., 2007). In vitro methods using epithelial cells have been beneficial in evaluating host-bacterial interactions and bacterial virulence (Elsinghorst, 1994; McCormick, 2003; Friis et al., 2005). Invasion into chicken epithelial cells by *Campylobacter* strains has been shown to be highly variable (Biswas et al., 2000; Carvalho et al., 2001; Hanel et al., 2004; Byrne et al., 2007). The

findings from the current study further suggest that *Campylobacter* invasion or dissemination, or both, into the tissues and organs does occur.

In summary, this study reveals the dynamic nature of *Campylobacter* spp. A high prevalence rate of *C. jejuni* and *C. coli* was observed in the 5 flocks evaluated from the 2 commercial laying operations. Presence in organs and tissues within Leghorn hens was similar to that found in other poultry even after several months in a caged environment. Tetracycline-resistant *Campylobacter* was most often observed. Surveys by public health authorities have led to table eggs not being regarded as a vehicle of foodborne *Campylobacter* infection, but it is still important to have an understanding of the ecology of these organisms in laying operations and the potential threat it may pose.

ACKNOWLEDGMENTS

We acknowledge Jeromey Jackson and Dianna Bourassa for their technical assistance with collection of samples. In addition, we thank Jodie Plumblee and Debbie Posey for their technical assistance with *Campylobacter* speciation and antimicrobial resistance profiling of isolates. All individuals were from the USDA, Agricultural Research Service. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the USDA.

REFERENCES

- Adesiyun, A., N. Offiah, N. Seepersadsingh, S. Rodrigo, V. Lashley, L. Musai, and K. Georges. 2005. Microbial health risk posed by table eggs in Trinidad. *Epidemiol. Infect.* 133:1049–1056.
- Allen, A. V., S. A. Bull, J. E. L. Corry, G. Domingue, F. Jorgensen, J. A. Frost, R. Whyte, A. Gonzalez, N. Elviss, and T. J. Humphrey. 2007. *Campylobacter* spp. contamination of chicken carcasses during processing in relation to flock colonization. *Int. J. Food Microbiol.* 113:45–61.
- Altkruse, S. F., N. J. Stern, P. I. Fields, and D. L. Swerdlow. 1999. *Campylobacter jejuni*—An emerging foodborne pathogen. *Emerg. Infect. Dis.* 5:28–35.
- Anonymous. 2009. Preliminary FoodNet data on the incidence of infection with pathogens transmitted commonly through food—10 States, 2008. *MMWR* 58:333–337.
- Avrain, L., C. Vernozy-Rozand, and I. Kempf. 2004. Evidence of natural horizontal transfer of *tetO* gene between *Campylobacter jejuni* strains in chickens. *J. Appl. Microbiol.* 97:134–140.
- Biswas, D., K. Itoh, and C. Sasakawa. 2000. Uptake pathways of clinical and healthy animal isolates of *Campylobacter jejuni* into INT-407 cells. *FEMS Immunol. Med. Microbiol.* 29:203–211.
- Black, R. E., M. M. Levine, M. L. Clements, T. P. Hughes, and M. J. Blaser. 1988. Experimental *Campylobacter jejuni* infection in humans. *J. Infect. Dis.* 157:472–479.
- Blaser, M. J. 1995. *Campylobacter* and related species. Pages 1948–1956 in *Principles and Practice of Infectious Diseases*. Churchill Livingstone, New York, NY.
- Blaser, M. J., J. L. Penner, and J. G. Wells. 1982. Diversity of serotypes involved in outbreaks of *Campylobacter* enteritis. *J. Infect. Dis.* 146:825–829.
- Buhr, R. J., N. A. Cox, N. J. Stern, M. T. Musgrove, J. L. Wilson, and K. L. Hiatt. 2002. Recovery of *Campylobacter* from segments of the reproductive tract of broiler breeder hens. *Avian Dis.* 46:919–924.
- Byrne, C., M. Clyne, and B. Bourke. 2007. *Campylobacter jejuni* adhere to and invade chicken intestinal epithelial cells in vitro. *Microbiology* 153:561–569.
- Camarda, A., D. G. Newell, R. Nasti, and G. Di Modugno. 2000. Genotyping *Campylobacter jejuni* strains isolated from the gut and oviduct of laying hens. *Avian Dis.* 44:907–912.
- Carvalho, A. C. T., G. M. Ruiz-Palacios, P. Ramos-Cervantes, L. E. Cervantes, X. Jiang, and L. K. Pickering. 2001. Molecular characteristics of invasive and noninvasive *Campylobacter jejuni* and *Campylobacter coli* isolates. *J. Clin. Microbiol.* 39:1353–1359.
- Cox, N. A., J. S. Bailey, L. J. Richardson, R. J. Buhr, D. E. Cosby, J. L. Wilson, K. L. Hiatt, G. R. Siragusa, and D. V. Bourassa. 2005. Presence of naturally occurring *Campylobacter* and *Salmonella* in the mature and immature ovarian follicles of late-life broiler breeder hens. *Avian Dis.* 49:285–287.
- Cox, N. A., L. J. Richardson, R. J. Buhr, P. J. Fedorka-Cray, J. S. Bailey, J. L. Wilson, and K. L. Hiatt. 2006a. Natural presence of *Campylobacter* spp. in various internal organs of commercial broiler breeder hens. *Avian Dis.* 50:450–453.
- Cox, N. A., L. J. Richardson, R. J. Buhr, J. K. Northcutt, J. S. Bailey, and P. J. Fedorka-Cray. 2007. Recovery of *Campylobacter* and *Salmonella* serovars from the spleen, liver/gallbladder, and ceca of six and eight week old commercial broilers. *J. Appl. Poult. Res.* 16:477–480.
- Cox, N. A., L. J. Richardson, R. J. Buhr, J. K. Northcutt, P. J. Fedorka-Cray, J. S. Bailey, B. D. Fairchild, and J. M. Mauldin. 2006b. Natural occurrence of *Campylobacter* spp., *Salmonella* serovars, and other bacteria in unabsorbed yolks of market-age commercial broilers. *J. Appl. Poult. Res.* 15:551–557.
- Cox, N. A., N. J. Stern, M. T. Musgrove, J. S. Bailey, S. E. Craven, P. F. Cray, R. J. Buhr, and K. L. Hiatt. 2002a. Prevalence and level of *Campylobacter* in commercial broiler breeder (parents) and broilers. *J. Appl. Poult. Res.* 11:187–190.
- Cox, N. A., N. J. Stern, J. L. Wilson, M. T. Musgrove, R. J. Buhr, and K. L. Hiatt. 2002b. Isolation of *Campylobacter* spp. from semen samples of commercial broiler breeder roosters. *Avian Dis.* 46:717–720.
- Doyle, M. P. 1984. Association of *Campylobacter jejuni* with laying hens and eggs. *Appl. Environ. Microbiol.* 47:533–536.
- Elsinghorst, E. A. 1994. Measurement of invasion by gentamicin resistance. *Methods Enzymol.* 236:405–420.
- Fallon, R., N. O'Sullivan, M. Maher, and C. Carroll. 2003. Antimicrobial resistance of *Campylobacter jejuni* and *Campylobacter coli* isolates from broiler chickens isolated at an Irish poultry processing plant. *Lett. Appl. Microbiol.* 36:277–281.
- Friedman, C. R., J. Neimann, H. C. Wegener, and R. V. Tauxe. 2000. Epidemiology of *C. jejuni* infections in the United States and other industrialized nations. Pages 121–138 in *Campylobacter*. 2nd ed. I. Nachamkin and M. J. Blaser, ed. ASM Press, Washington, DC.
- Friis, L. M., C. Pin, B. M. Pearson, and J. M. Wells. 2005. In vitro cell culture methods for investigating *Campylobacter* invasion mechanisms. *J. Microbiol. Methods* 61:145–160.
- Ge, B., D. G. White, P. F. McDermott, W. Girard, S. Zhao, S. Hubert, and J. Meng. 2003. Antimicrobial-resistant *Campylobacter* species from retail raw meats. *Appl. Environ. Microbiol.* 69:3005–3007.
- Greig, J. D., and A. Ravel. 2009. Analysis of foodborne outbreak data reported internationally for source attribution. *Int. J. Food Microbiol.* 130:77–87.
- Gupta, A., J. M. Nelson, T. J. Barrett, R. V. Tauxe, S. P. Rossiter, C. R. Friedman, K. W. Joyce, K. E. Smith, T. F. Jones, M. A. Hawkins, B. Shiferaw, J. L. Beebe, D. J. Vugia, T. Rabatsky-Her, J. A. Benson, T. P. Root, and F. J. Angulo. 2004. Antimicrobial resistance among *Campylobacter* strains, United States, 1997–2001. *Emerg. Infect. Dis.* 10:1102–1109.
- Gyles, C. L. 2008. Antimicrobial resistance in selected bacteria from poultry. *Anim. Health Res. Rev.* 9:149–158.
- Hanel, I., J. Muller, W. Muller, and F. Schulze. 2004. Correlation between invasion of Caco-2 eukaryotic cells and colonization ability in the chick gut in *Campylobacter jejuni*. *Vet. Microbiol.* 101:74–82.
- Hiatt, K. L., N. J. Stern, P. Fedorka-Cray, N. A. Cox, and B. S. Seal. 2007. Molecular phylogeny of the *flaA* short variable region

- among *Campylobacter jejuni* isolates collected during an annual evaluation of poultry flocks in the southeastern United States. *Foodborne Pathog. Dis.* 4:339–347.
- Humphrey, T. J., S. O'Brien, and M. Madsen. 2007. *Campylobacter* as zoonotic pathogens: A food production perspective. *Int. J. Food Microbiol.* 117:237–257.
- Jacobs-Reitsma, W. F. 1997. Aspects of epidemiology of *Campylobacter* in poultry. *Vet. Q.* 19:113–117.
- Jacobs-Reitsma, W. F. 2000. *Campylobacter* in the food supply. Pages 467–481 in *Campylobacter*. I. Nachamkin and M. J. Blaser, ed. American Society for Microbiology, Washington, DC.
- Jones, D. R., and M. T. Musgrove. 2007. Pathogen prevalence and microbial levels associated with restricted shell eggs. *J. Food Prot.* 70:2004–2007.
- Keener, K. M., M. P. Bashor, P. A. Curtis, B. W. Sheldon, and S. Kathariou. 2004. Comprehensive review of *Campylobacter* and poultry processing. *Compr. Rev. Food Sci. Food Saf.* 3:105–116.
- Lastovica, A. J. 2006. Emerging *Campylobacter* spp.: The tip of the iceberg. *Clin. Microbiol. Newsl.* 28:49–55.
- Luangtongkum, T., Y. Morishita, A. J. Ison, S. Haug, P. F. McDermott, and Q. Zhang. 2006. Effect of conventional and organic production practices on the prevalence and antimicrobial resistance of *Campylobacter* spp. in poultry. *Appl. Environ. Microbiol.* 72:3600–3607.
- Luangtongkum, T., Y. Morishita, L. Martin, I. Choi, O. Sahin, and Q. Zhang. 2008. Prevalence of tetracycline-resistant *Campylobacter* in organic broilers during a production cycle. *Avian Dis.* 52:487–490.
- McCormick, B. A. 2003. The use of transepithelial models to examine host-pathogen interactions. *Curr. Opin. Microbiol.* 6:77–81.
- Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin, and R. V. Tauxe. 1999. Food-related illness and death in the United States. *Emerg. Infect. Dis.* 5:607–625.
- Meinersmann, R. J., L. O. Helsel, P. I. Fields, and K. L. Hiett. 1997. Discrimination of *Campylobacter jejuni* strains by *fla* gene sequencing. *J. Clin. Microbiol.* 35:2810–2814.
- Meinersmann, R. J., W. E. Riggsby, N. J. Stern, L. C. Kelley, J. E. Hill, and M. P. Doyle. 1991. Comparative study of colonizing and noncolonizing *Campylobacter jejuni*. *Am. J. Vet. Res.* 52:1518–1522.
- Nauta, M. J., and A. H. Havelaar. 2008. Risk-based standards for *Campylobacter* in the broiler meat chain. *Food Contr.* 19:372–381.
- Papadopoulos, C., D. Dimitriou, S. Levidiotou, H. Gessouli, A. Panagiotou, S. Golegou, and G. Antoniadou. 1997. Bacterial strains isolated from eggs and their resistance to currently used antibiotics: Is there a health hazard for consumers? *Comp. Immunol. Microbiol. Infect. Dis.* 20:35–40.
- Piddock, L. J. V., V. Ricci, K. Stanley, and K. Jones. 2000. Activity of antibiotics used in human medicine for *Campylobacter jejuni* isolated from farm animals and their environment in Lancashire, U.K. *J. Antimicrob. Chemother.* 46:303–306.
- Price, L. B., E. Johnson, R. Vailes, and E. Silbergeld. 2005. Fluoroquinolone-resistant *Campylobacter* isolates from conventional and antibiotic-free chicken products. *Environ. Health Perspect.* 113:557–560.
- Randall, L. P., A. M. Ridley, S. W. Cooles, M. Sharma, A. R. Sayers, L. Pumbwe, D. G. Newell, L. J. V. Piddock, and M. J. Woodward. 2003. Prevalence of multiple antibiotic resistances in 443 *Campylobacter* spp. isolated from humans and animals. *J. Antimicrob. Chemother.* 52:507–510.
- Richardson, L. J., N. A. Cox, J. S. Bailey, P. J. Fedorka-Cray, M. E. Berrang, M. A. Harrison, and J. M. Cox. 2009. Evaluation of TECRA[®] broth, Bolton's broth and direct plating for recovery of *Campylobacter* spp. from broiler carcass rinses from several commercial processing plants. *J. Food Prot.* 72:972–977.
- Rivoal, K., C. Ragimbeau, G. Salvat, P. Colin, and G. Ermet. 2005. Genomic diversity of *Campylobacter coli* and *Campylobacter jejuni* isolates recovered from free-range broiler farms and comparison with isolates of various origins. *Appl. Environ. Microbiol.* 71:6216–6227.
- Shane, S. M., and N. J. Stern. 2003. *Campylobacter* infection. Pages 620–622 in *Diseases of Poultry*. 11th rev. ed. Blackwell Publishing, Ames, IA.
- Solomon, E. B., and D. G. Hoover. 1999. *Campylobacter jejuni*: A bacterial paradox. *J. Food Saf.* 19:121–136.
- Son, I., M. D. Englen, M. E. Berrang, P. J. Fedorka-Cray, and M. A. Harrison. 2007. Antimicrobial resistance of *Arcobacter* and *Campylobacter* from broiler carcasses. *Int. J. Antimicrob. Agents* 29:451–455.
- Suzuki, H., and S. Yamamoto. 2009. *Campylobacter* contamination in retail poultry meats and by-products in the world: A literature survey. *J. Vet. Med. Sci.* 71:255–261.
- Tauxe, R. V. 1992. Epidemiology of *Campylobacter jejuni* infections in the United States and other industrialized nations. Pages 9–30 in *Campylobacter jejuni*: Current Status and Future Trends. 1st ed. American Society for Microbiology, Washington, DC.
- Taylor, D. E., and P. Courvalin. 1988. Mechanisms of antibiotic resistance in *Campylobacter* species. *Antimicrob. Agents Chemother.* 32:1107–1112.